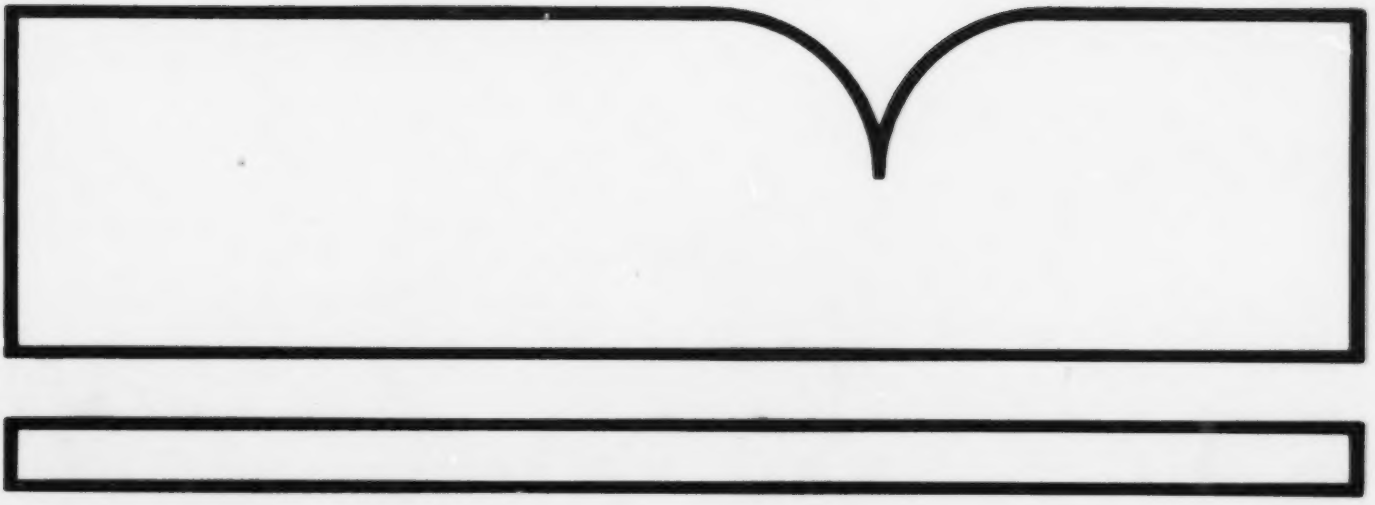
PB81-213167

Preparation of Extrapulmonary Tissues and Body Fluids for Quantitative Transmission Electron Microscope Analysis of Asbestos and Other Mineral Particle Concentrations

(U.S.) Environmental Research Lab.
Duluth, MN

1979



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TECHNICAL REPORT DATA
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1. REPORT NO.	2.	3.
EPA-600/J-79-123	Journal Article	PB81-213167

4. TITLE AND SUBTITLE
Preparation of Extrapulmonary Tissues and Body Fluids for Quantitative Transmission Electron Microscope Analysis of Asbestos and Other Mineral Particle Concentrations

5. REPORT DATE

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
Philip M. Cook

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
U.S. Environmental Protection Agency
Environmental Research Laboratory-Duluth
6201 Congdon Boulevard
Duluth, Minnesota 55804

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Same as above

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA-600/03

15. SUPPLEMENTARY NOTES IN: Annals of the New York Academy of Sciences, Volume 330, pages 717-724, December 14, 1979

16. ABSTRACT

The preparation of tissue and body fluid samples is a critical step in the determination of microscopic particle concentrations and characteristics by electron microscopy. A critical review of tissue preparation techniques reported in the literature is provided with emphasis on analytical sensitivity and accuracy and the problem of particle contamination. Details of a method devised for analysis of mineral fibers in urine samples are provided as an example of how the basic requirements for insuring quantitative particle analysis are achieved.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

PRODUCT OF:
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	18
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

Reprinted from
ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume 330 Pages 717-724
December 14,1979 27052

Workshop: Demonstration, Identification and Quantification of Asbestos in Tissues

EPA-600/J-79-123
Journal Article

PREPARATION OF EXTRAPULMONARY TISSUES AND BODY FLUIDS FOR QUANTITATIVE TRANSMISSION ELECTRON MICROSCOPE ANALYSIS OF ASBESTOS AND OTHER MINERAL PARTICLE CONCENTRATIONS

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Exposure of the human body to microscopic mineral particles by inhalation and/or ingestion may result in the retention of potentially pathogenic particles in tissues. Most particles are eliminated, however, by various physiological mechanisms that operate to clear the body of foreign materials.

The transmission electron microscope analysis of tissue samples for fine particle content provides a direct measurement of cumulative particle retention, size distribution, and particle migration routes. This information can ultimately by used as an index of exposure and the incidence of disease thus related to the amount, type, and size of particles accumulated in the tissue. Scanning electron microscope study of particles in tissue can provide additional information about the three-dimensional relationship of particles with cellular structure.[1,2]

The detection of asbestos fibers and other inorganic particles in human pulmonary tissues has been reported by many investigators. Several basic techniques have been used to prepare lung tissue for transmission electron microscope examination. Particles have been examined *in situ*[3,4,5] in thin tissue sections although surrounding tissue can interfere with observation and identification. Particle losses and inopportune particle orientations are additional problems of tissue section analysis.[6]

The often used carbon extraction technique [7,8] involves ashing (450–500° C) a thin (approximately 6 μm) tissue section on a glass slide. A plastic film is hardened from polyvinyl alcohol placed on the sample ash. The plastic film with embedded ashed sample residue is peeled off the slide and the tissue side coated with a layer of carbon. Small pieces of the carbon film, which contains the sample ash, are floated on water and then placed on electron microscope grids. A distinct advantage of this technique is the ability to observe particles with the original tissue sample anatomical distribution preserved.

Larger tissue samples can be digested with 5 percent potassium hydroxide solution[9,10] at 90° C for 4 hours. The residue is washed with particle-free water and centrifuged for several cycles to remove excess KOH and digested materials. The residue is then dispersed in particle-free water by sonification and a drop is placed on a carbon-coated electron microscope grid and dried.

Another chemical digestion technique involves 5 percent sodium hypochlorite treatment[11] at approximately 60° C. The resultant solution is centrifuged and the sediment washed several times with water. A drop of the final suspension is placed on a carbon-coated electron microscope grid and dried. A superior version[12,13] of the chemical digestion techniques involves filtering the digested tissue suspension through a Nuclepore* membrane filter which is subsequently carbon-coated. Electron micro-

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0077-8923/79/0330-0717 [redacted] 1979, NYAS

scope grids are prepared by dissolving filter pieces in chloroform as described later in this report. The elimination of repeated washing and centrifugation steps avoids loss of sample and minimizes the introduction of contaminant particles during sample preparation.

High temperature ashing of bulk tissue samples may result in sample loss, decomposition of minerals, and severe clumping of the resultant ash particles. Low temperature ashing in an oxygen plasma followed by treatment of the ash with hydrochloric acid allows the filtration of a concentrated ash suspension on a membrane filter.[14] The filter may then be carbon-coated and the direct transfer method used to prepare electron microscope grids.

Variations[15,16] of the four basic tissue preparation techniques described above include combined chemical digestion and ashing, use of different membrane filters, preparation of grids from a drop of final suspension, repeated ashing steps, etc. Almost no data have been reported on the accuracy, precision, or amount of particle contamination possible for the various techniques reported. This absence of quality control data is explained in part by the tendency of many studies to involve only qualitative examination of tissue particle content. Since risk of disease is likely to be related in part to the number of particles accumulated in tissue, much work is needed on the evaluation of quantitative tissue analysis techniques.

Requirements for Quantitative Electron Microscope Analysis of Extrapulmonary Tissue for Fine Particles

Selection of tissue samples for particle analysis is based on the organs and specific tissue types that are physiologically important to the investigation. Exposure, bodily transport, and elimination mechanisms interact over time to produce the particle distribution found in tissues at death. The tissue sampling strategy should also consider the often nonrandom distribution of particles in tissue of interest.

The ideal tissue analysis technique will provide information about the distribution, concentration, and characteristics of particles in the original tissue sample. To accomplish this, the technique must:

1. Obtain a sample preparation on an electron microscope grid suitable for observation and identification of particles (uncrowded, uniform distribution of particles);
2. Achieve a realistic detection limit that allows measurement of particle concentrations of interest in a reasonable amount of microscope time;
3. Retain the original particle size distribution and physical and chemical characteristics;
4. Prevent or minimize the introduction of particle contamination;
5. Allow selected area electron diffraction and energy dispersive x-ray identification of particles;
6. Utilize good counting and particle sizing statistics;
7. Utilize the minimum number of steps necessary to prepare electron microscope grids from tissue samples;
8. Provide acceptable levels of accuracy and precision.

Sensitivity

Analysis of extrapulmonary tissues and fluids requires special consideration of sensitivity. The attainment of minimum detection limits consistent with fulfillment of

other analysis requirements is essential. Consider the case of amphibole fiber contamination of the Duluth, Minnesota municipal water supply.[17,18] The drinking water contained a concentration of approximately 50×10^6 amphibole fibers per liter averaged over a period of 20 years (higher concentrations in later years) prior to establishment of a highly effective filtration plant in 1977. Persons ingesting the water had no other exposure to these amphibole fibers. Electron microscope examination of tissues from deceased residents of Duluth might therefore provide valuable information about the ability of ingested fibers to pass the gastrointestinal mucosa and accumulate in the body.

What tissue preparation technique would be appropriate for examining liver, spleen, mesenteric lymph node, kidney, etc. tissues of Duluth residents whose total 20 year exposure is approximately 7.3×10^{11} amphibole fibers (assuming an average daily water ingestion of two liters)? In order to estimate a possible tissue fiber concentration, assume that one in ten thousand ingested fibers passes the gastrointestinal mucosa and is retained indefinitely in certain tissues that represent one-tenth of the total body weight. A subject weighing 150 pounds would therefore accumulate 7.3×10^7 fibers in 15 pounds of tissue after 20 years or about 5,000 fibers per gram or cubic centimeter of affected tissue.

The carbon extraction tissue preparation technique, which has been attempted for some extrapulmonary tissue analyses, provides an electron microscope grid containing a sample representing an approximately 6 μm thick tissue section. A typical 200 mesh locator grid has openings of about 9,000 μm^2 in area so one grid opening represents approximately 54,000 μm^3 of tissue. Such a volume of tissue for the Duluth tissue concentration estimate would contain 2.7×10^{-4} fibers. In other words, we can expect to find one amphibole fiber in 3700 grid squares; an impossible task since thorough electron microscope examination of each grid square area should require from 15 to 60 minutes depending on how much debris and other particles are present.

A more favorable detection limit is attained by chemical digestion and/or low temperature ashing a larger piece of tissue. Assume that 2 cm^3 of tissue (approximately 10,000 amphibole fibers) can be processed and filtered on a 25 mm membrane filter (250 mm^2 filtration area). Direct transfer preparation of electron microscope grids should result in the observation of one amphibole fiber for every 2.8 grid squares examined. A good level of precision for determination of the fiber concentration (assuming a Poisson distribution) might require identification of 20 fibers or examination of 56 grid squares, a very time consuming and thus expensive analysis. To the extent a larger piece of tissue can be processed without producing grids too crowded with debris, the analysis time can be shortened.

Accuracy

Much of the recent research on improved electron microscope asbestos fiber analysis techniques for water and air samples provides information applicable to tissue analysis. The preparation of electron microscope grids from samples on membrane filters can result in large, variable losses of particles.[19,20] An excellent technique for preventing such loss involves the filtration of the sample on a Nuclepore® filter (FIGURE 1). The filter is carbon-coated so that the particles on the very smooth filter surface are embedded in the carbon film. Small pieces (~1 mm^2) of the carbon-coated filter are placed, carbon side down, on electron microscope grids resting on a wire screen on top of a pad of absorbent material such as a stack of filter paper in a glass petri dish. A 10 μl drop of chloroform fixes each filter piece to its grid and initiates dissolution of the filter. The petri dish is immediately filled with chloroform until the

solvent level reaches the wire screen and then the petri dish is covered and the solvent level maintained. The filter is normally completely dissolved after 4–12 hours of this wicking solvent action. This direct transfer method produces electron microscope grids supporting carbon films that contain a uniform distribution of particles representative of the original sample particle concentration. This allows the small portion of sample

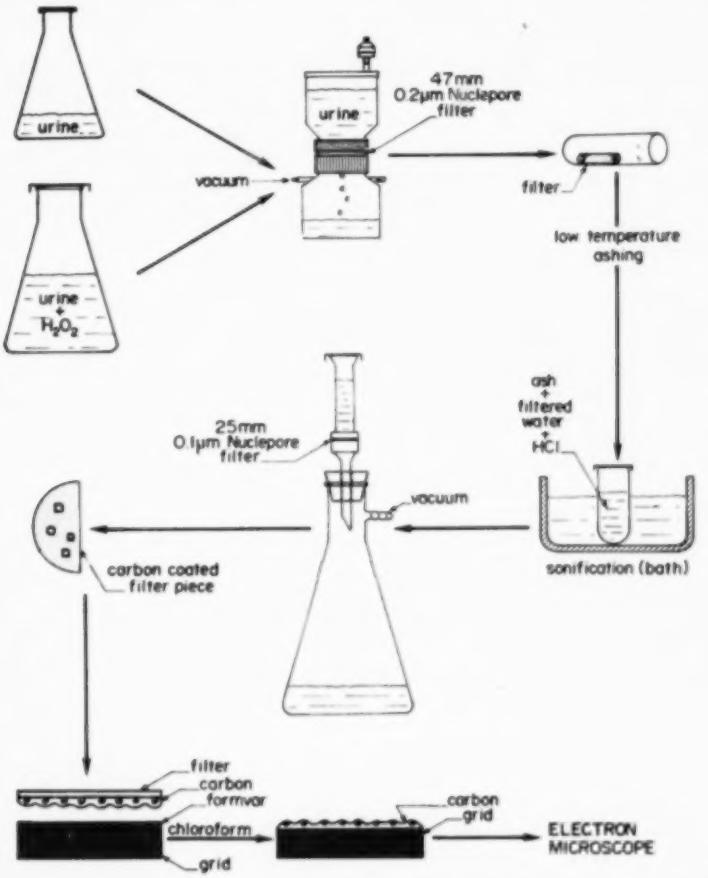

FIGURE 1. Preparation of electron microscope grids for quantitative determination of urine sample inorganic particle concentrations.

actually observed on the electron microscope to produce an accurate calculation of particle concentration.

Particle loss can occur during other sample preparation steps. Little or no information exists for the magnitude of such losses during tissue analysis. Repeated centrifugation and washing steps should be avoided since chemical destruction of

particles is possible. Clumping of particles results in difficulty in microscope observation and identification and thus may cause an observer to underestimate particle concentration. Aliquot removal from inhomogeneous particle suspensions can also contribute to inaccuracy. Particle size and interferring debris and organic material contribute to the observer's inability to identify particles by selected area electron diffraction and energy dispersive x-ray analysis.

A potential cause of asbestos fiber concentration overestimation is the use of ultrasonic energy to disperse particles in water after ashing or chemical digestion. Fiber bundles can break down into smaller fragments or individual fibrils when ultrasonic energy of sufficient power and duration is applied. Fibrous amphibole particles are much less susceptible to such disintegration than chrysotile which is reported[20] to require more than 0.5 watt/ml ultrasonic power for any breakdown to occur. Bath type sonification can be used to disperse fibers without increasing the number of fibers. Incomplete dispersal of fibers results in concentration underestimates due, in part, to particle clumping that obscures individual fibers. Tissue or body fluid preparation procedures involving ultrasonification should be evaluated by analysis of samples spiked with known amounts of the mineral particles of interest.

Contamination

Another possible source of particle concentration overestimation is the addition of contamination during sample preparation. Many particles of interest are unlikely to present a problem if an effort is made to use particle-free glassware; water and solvents used in cleaning and sample preparation are well filtered; and the sample is protected from dustfall. Chrysotile asbestos is perhaps the most persistent contaminant because of its small fibril size, ubiquity in the environment, and use in many building construction products. All tissue analyses should be accompanied by blank sample analysis in addition to control tissue samples in order to determine if contamination is present. The blank sample must be processed through each step of the sample preparation procedures along with the tissue samples.

Use of clean room techniques and other precautions do not necessarily insure the prevention of contamination. Membrane filters are usually contaminated with chrysotile. Analysis of low temperature ashed filters compared to carbon-coated blank filter grid preparations indicates that most fibers are present within the membrane and not resting on its surface. Thus very little contamination results from use of membrane filters to prepare electron microscope grids. The analysis of samples ashed with a membrane filter, however, must consider how many fibers are introduced from the ashed filter. Since filter contamination can vary considerably from batch to batch, blank filter analysis should be accomplished for each package of filters used. Crocidolite fiber contamination was recently found in a batch of membrane filters as a result of the manufacturer purchasing filter material from a new source.

Analysis of Urine Samples

The previously discussed case of amphibole fiber contaminated drinking water pointed out the difficulty of detecting the fibers in the tissue of deceased individuals who drank the water. Body fluid samples collected from individuals regularly ingesting these fibers might contain more detectable fiber concentrations because fiber loss by various elimination mechanisms would be balanced against fiber accumulaion due to continued ingestion exposure.

Attempted preparation of a whole blood sample by low temperature ashing

resulted in insufficient sensitivity for electron microscope examination due to interfering debris. The possibility that examination of human urine might provide evidence for passage of ingested fibers through the gastrointestinal mucosa seemed worth examination since the daily production of urine represents several hundred passes of the body's blood through the kidneys. In addition to a possible higher concentration of fibers in urine than blood, a much greater volume of urine was thought likely to be preparable for electron microscope examination. Urine samples from individuals ingesting amphibole fibers in drinking water were found to contain amphibole fibers while no amphibole fibers could be found in the urine of individuals with no history of exposure to amphibole fibers.[23] Urine amphibole fiber concentrations appear to be greatly reduced within a few months after ingestion of fibers ceases.

The procedure devised for preparation of urine samples for electron microscopy is summarized in FIGURE 1. Samples are collected in clean wide-mouth glass Erhlenmeyer flasks and immediately recapped with aluminum foil. Contact of skin or clothes with the flask opening is prevented and the first approximately 20 ml of urine is discarded to eliminate any chance of sample contamination. All glassware used in sample collection or preparation is thoroughly scrubbed and rinsed several times with distilled water that has been filtered successively through 0.45 μm and 0.1 μm membrane filters.

Urine samples ranging in volume up to 300 ml can be directed filtered through a 47 mm 0.2 μm Nuclepore* filter. The urine sample should be immediately filtered after collection with an apparatus (FIGURE 1) that provides a filtered air environment during the very slow filtration. Precipitates can often be dissolved by adding a few drops of 1N HCl. Larger volume urine samples may be digested with approximately 20 ml of 30 percent hydrogen peroxide and heat for 12 or more hours prior to filtration. Peroxide treatment allows for the faster filtration of larger volumes of urine but does not remove all interfering organic matter.

The membrane filter with urine sediment is placed in a covered plastic petri dish, dried at 70° C, and then stored in a refrigerator. Later the entire filter is low temperature ashed in an oxygen plasma. The ash is resuspended in approximately 20 ml of filtered distilled water in the same tube used for ashing the filter. A few drops of 1N HCl are added to insure dissolution of inorganic salts. The suspension is dispersed by swirling and five minutes of bath ultrasonification (low energy).

The entire ash suspension is filtered through a 25 mm 0.1 μm Nuclepore filter. The use of a 25 mm filter results in a four-fold greater particle density than on the original 47 mm filter. The 25 mm filter is carbon-coated to a thickness of about 10 nm and electron microscope grids prepared with the chloroform wicking procedure described previously.

Examination of urine sample grids is relatively easy for samples with the highest amphibole fiber concentrations (>100 fibers/ml). Samples with lower fiber numbers require long periods of electron microscope examination to determine the fiber concentration. Since well-ashed samples provide grids without particle crowding, further sample concentration may be possible in order to obtain more fibers per area examined on the grids. Hydrogen peroxide treatment of larger volumes of urine would allow an improvement in analytical sensitivity.

Elimination of membrane filtration of the urine sample prior to low temperature ashing would prevent chrysotile fiber contamination from the ashed membrane filter. Complete chemical digestion of all organic matter in urine might allow simple membrane filtration followed by direct transfer to an electron microscope grid. Ozone

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gas and ultraviolet light treatment of wine and beer samples[20] allows such a single filtration technique and will be attempted for urine samples.

Replicate analysis of urine samples has not been attempted to date due to the loss of analysis sensitivity produced by subdividing a sample. Ninety-five percent confidence intervals can be calculated on the basis of a Poisson distribution for each concentration determined.[22] Urine samples with nondetectable amphibole fiber concentrations were spiked with a measured volume of water containing amphibole fibers. Electron microscope determined fiber concentrations for the spiked urine samples and the amphibole fiber water suspension indicate a 29 percent underestimate of amphibole fibers for the urine sample preparations. This modest "loss" of fibers is consistent with results obtained for low temperatures ashed air particulate samples on membrane filters. The tendency to underestimate fiber concentration may best be explained by failure to disperse all clumps of ashed urine sediment and inability to identify fibers in the urine samples due to debris not present as an interference in the water-amphibole fiber suspension.

Conclusions

Once tissue or body fluid samples are transferred to electron microscope grids, the procedures for sizing, counting, and identifying particles are the same as those used for analysis of air and water samples. Considerable discussion of these techniques exists in the recent literature and much research is currently directed toward improving and standardizing procedures.

Tissue sample preparation techniques must be evaluated for their ability to provide electron microscope grids with particle numbers and characteristics unchanged from the original tissue. Only when such preparations are achieved can the measured particle concentration, size distribution, etc., be correlated to other parameters such as incidence of disease and exposure history. Even the qualitative assessment of the presence of particles such as chrysotile fibrils requires confidence in their tissue, rather than contamination origin. Such confidence can often only be obtained through quantitative particle concentration analysis of tissue and blank samples.

References

1. Dense, P. B., J. L. Abraham & A. H. Gelderman. 1973. Methods for a SEM study of coal worker's pneumoconiosis. Scanning Electron Microsc. (Part III): 411–418.
2. Dense, P. B., R. G. Frederickson & R. S. Pope. 1977. Heavy metal staining of paraffin, epoxy, and glycol methacrylate embedded biological tissue for scanning electron microscope histology. Scanning Electron Microsc. II: 83–92.
3. Henderson, W. J. 1969. A simple replication technique for the study of biological tissues by electron microscopy. J. Microsc. 89: 369–372.
4. Hitchins, M., N. H. Hodgkin & A. Tanaka. 1974. Japanese gastric cancer: potentially carcinogenic silicates (talc) from rice. Arch. Pathol. 97: 366–368.
5. Westlake, G. E., H. J. Spurt & M. N. Smith. 1985. Penetration of colonic mucosa by asbestos particles: an electron microscopic study in rats fed asbestos dust. Lab. Invest. 16(11): 2029–2033.
6. Stewart, I. M. 1978. Tissue preparation for mineral analysis. *In*: Electron Microscopy and X-Ray Applications to Environmental and Occupational Health. P. A. Russell & A. E. Hutchings, Eds.: 229–235. Ann Arbor Science Publishers, Ann Arbor, Mich.
7. Langer, A. M., I. B. Rubin, I. J. Selikoff & F. Pooley. 1972. Chemical characterization of uncoated asbestos fibers from the lungs of asbestos workers by electron microprobe analysis. J. Histochem. Cytochem. 20(9): 735–740.

8. POOLEY, F. D. 1976. An examination of the fibrous mineral content of asbestos lung tissue from the Canadian chrysotile mining industry. Environ. Res. **12**: 281–298.
9. LANGER, A. M., I. J. SELIKOFF & A. SASTRE. 1971. Chrysotile asbestos in the lungs of persons in New York City. Arch. Environ. Health. **22**: 348–361.
10. POOLEY, F. D. 1971. Electron microscope characteristics of inhaled chrysotile asbestos fibre. Br. J. Ind. Med. **29**: 146–153.
11. GROSS, P., R. A. HARLEY, L. M. SWINBURNE & J. M. DAVIS. 1974. Ingested mineral fibers: do they penetrate tissue or cause cancer? Arch. Environ. Health. **29**: 341–347.
12. JAURAND, M. C., J. BIGNON, P. SEBASTIEN & J. GONI. 1977. Leaching of chrysotile asbestos in human lungs. Environ. Res. **14**: 245–254.
13. SEBASTIEN, P., X. JANSON, G. RIBA, R. MASSE, G. BONNAUD & J. BIGNON. 1978. Translocation of asbestos fibers through respiratory tract and gastrointestinal tract according to fiber type and size. *In* Dust and Disease: Occupational and Environmental Exposures to Selected Fibrous and Particulate Dust. J. Dement & R. Lemen, Eds. Society for Occupational and Environmental Health, Washington, D.C.
14. LIBOUTANT, L. 1974. Investigation and analysis of asbestos fibers and accompanying minerals in biological materials. Environ. Health Perspect. **9**: 149–153.
15. CUNNINGHAM, H. M., C. A. MOODIE, G. A. LAWRENCE & R. D. PONTEFRACT. 1977. Chronic effects of ingested asbestos in rats. Arch. Environ. Contam. Toxicol. **6**: 507–513.
16. CUNNINGHAM, H. M., R. D. PONTEFRACT & R. C. O'BRIEN. 1976. Quantitative relationship of fecal asbestos to asbestos exposure. J. Toxicol. Environ. Health. **1**: 377–379.
17. COOK, P. M., G. E. GLASS & J. H. TUCKER. 1974. Asbestiform amphibole minerals: detection and measurement of high concentrations in municipal water supplies. Science **185**: 853–855.
18. COOK, P. M., I. B. RUBIN, C. J. MAGGIORE & W. J. NICHOLSON. 1976. X-ray diffraction and electron beam analysis of asbestiform minerals in Lake Superior waters. Proc. Int. Conf. Environ. Sensing and Assess. **2**(34–1): 1–9. Institute of Electrical and Electronics Engineers, New York, N.Y.
19. BEAMAN, D. R. & D. M. FILE. 1976. Quantitative determination of asbestos fiber concentrations. Anal. Chem. **48**(1): 101–110.
20. CHATFIELD, E. J. & M. J. DILLON. 1978. Some aspects of specimen preparation and limitations of precision in particulate analysis by sem and tem. Scanning Electron Microsc. Vol. I.: 487–496.
21. COOK, P. M. & G. F. OLSON. 1978. Human urine elimination of ingested mineral fibers. Science **204**: 195–198.
22. HALLENBECK, W. H., E. H. CHEN, K. PATEL-MANDLIK & A. H. WOLFF. 1977. Precision of analysis for waterborne chrysotile asbestos by transmission electron microscopy. Bull. Environ. Contam. Toxicol. **17**(5): 551–558.